Characterization and splice variants of neuronal nitric oxide synthase in rat small intestine

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Huber, Andrea, Dieter Saur, Manfred Kurjak, Volker Schusdziarra, and Hans-Dieter Allescher. Characterization and splice variants of neuronal nitric oxide synthase (nNOS) in rat small intestine. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1146–G1156, 1998.—The aim of this study was to characterize neuronal nitric oxide synthase (nNOS) activity and 5'-end splice variants in rat small intestine. nNOS was predominantly expressed in the longitudinal muscle layer, with attached myenteric plexus (LM-MP). The biochemical properties of NOS activity in enriched nerve terminals resemble those of nNOS isolated from the brain. Western blot analysis of purified NOS protein with an nNOS antibody showed a single band in the particulate fraction and three bands in the soluble fraction. Rapid amplification of 5' cDNA ends-PCR revealed the presence of three different 5'-end splice variants of nNOS. Two variants encode for nNOSα, which has a specific domain for membrane association. The third variant encodes for nNOSβ, which lacks the domain for membrane association and should therefore be soluble. nNOS is predominantly expressed in LM-MP and can be enriched in enteric nerve terminals. We present the first evidence that three 5'-end splice variants of nNOS encoding two different proteins are expressed in rat small intestine. These two NOS enzymes exhibit different subcellular locations and might be implicated in different biological functions.

NITRIC OXIDE (NO) is a nonadrenergic, noncholinergic (NANC) neurotransmitter that has a potent inhibitory effect on the smooth muscle in various regions of the gastrointestinal tract (11, 41, 43). NO is generated from the amino acid L-arginine and may also act as a neuromodulator by facilitation or inhibition of the release of other neurotransmitters within the same nerve ending or as a neurotransmitter acting on enteric neurons or smooth muscle cells of the gastrointestinal tract (2). Also, there is some evidence (36, 40, 46) that NO might act as a second messenger within smooth muscle or interstitial cells of Cajal.

Three distinct isoforms of NO synthase (NOS) have been purified and cloned: an inducible NOS (iNOS) (48), which is Ca2+-independent, and an endothelial NOS (eNOS) (35) and a neuronal NOS (nNOS) (6, 13), which are both Ca2+-dependent. Recent evidence (8, 15) from the mouse brain suggests that alternative 5'-end splicing of NOS mRNA results in at least three different NH2-terminal nNOS protein variants (nNOSα, nNOSβ, and nNOSγ). nNOSα, which exhibits full enzymatic activity, contains a PDZ (PSD-95/Dlg/ZO-1) domain enabling a possible interaction with proteins of the postsynaptic density (PSD) zone, such as postsynaptic density protein-95 (PSD-95) or α-syntrophin. Because of this interaction, the nNOSα form can be membrane associated (8). The nNOSβ and nNOSγ splice variants lack the PDZ domain that is encoded by exon 2. This results in a cytosolic localization of these proteins. nNOSβ and nNOSγ from the mouse central nervous system showed an enzymatic activity of ~80% and ~3%, respectively, of that of nNOSα, when ex- pressed in mammalian cells (8). In addition, in the rat three different splice forms with distinct 5'-untrans- lated first exons (5'-untranslated region) of the nNOSα mRNA (nNOSα-a, nNOSα-b, nNOSα-c) have been identified, which show a tissue- and development-specific expression (31). However, these splice forms result in a single nNOSα protein.

Recently, it has been shown (45) that NOS expression in the rat stomach can be changed substantially under pathophysiological conditions, such as diabetic autonomic neuropathy, causing defective relaxation in response to NANC nerve stimulation.

In the present study, we quantified the distribution of NOS mRNA expression in various layers of rat small intestine and characterized the biochemical properties of NOS in enriched enteric nerve terminals. Subsequently, we analyzed purified NOS by Western blot analysis and investigated the expression of possible 5'-end nNOS splice variants by rapid amplification of 5' cDNA ends-PCR (5'-RACE-PCR).

MATERIALS AND METHODS

Preparation of rat enteric nerve terminals. The preparation of rat enteric nerve terminals was carried out as described previously (28). Briefly, male Wistar rats were killed by cervical dislocation. The small intestine was suspended in ice-cold sucrose-MOPS buffer [25 mM MOPS, pH 7.4, 10 mM MgCl2, and 8% (wt/vol) sucrose], stripped of mesenteric arcade and fat, and opened along the mesenteric attachment line, and the mucosal layer was removed by scraping. The remaining muscle layers were minced with scissors and homogenized with a Polytron PT20 homogenizer at a setting of ~1,500 rpm for 15 s (3 times for 5 s each). The tissue homogenate was centrifuged at 800 g for 10 min, and the supernatant was collected [postnuclear supernatant (PNS)] and subjected to various differential centrifugation steps (3,500 g for 10 min, 120,000 g for 60 min, and 10,000 g for 10 min). The resulting pellet of the final centrifugation step was the enriched nerve terminals (P2). Protein concentrations were determined according to the method of Bradford (4).
using bovine plasma γ-globulin as a standard (Bio-Rad, Munich, Germany). For a single preparation, the small intestines of five rats were used. The detailed characterization of the enriched enteric nerve terminals and the method of [3H]axotocin binding have been described previously (1, 28). Release of bombesin-, somatostatin-, and vasoactive intestinal polypeptide (VIP)-like immunoreactivity from enriched enteric nerve terminals was analyzed by RIA, as reported previously (2, 28, 29).

**Extraction of proteins.** Tissue from both rat brain and rat small intestinal longitudinal muscle layer, with attached myenteric plexus (LM-MP), was cut into pieces and homogenized with a fivefold volume of buffer [50 mM Tris, pH 7.6, 0.1 mM EDTA, 0.1 mM EGTA, 3 µM leupeptin, 1 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 12 mM β-mercaptoethanol] with a Polytron PT20 homogenizer at about 1,500 rpm for 15 s (3 times for 5 s each). The homogenates were centrifuged at 100,000 g for 60 min, and the resulting supernatants were referred to as soluble brain and small intestinal fractions (BRs and SI(s), respectively). The pellets were suspended with the same buffer and subsequently centrifuged (60,000 g for 60 min). To obtain the particulate fraction, we homogenized the resulting pellets with buffer containing 1% Triton X-100 and centrifuged them at 100,000 g for 60 min. The supernatants [particulate brain and small intestinal fractions (BRp and SIp), respectively] were then collected for further analysis.

**Enzyme purification.** NOS was isolated and purified as previously reported (42). Briefly, the enriched nerve terminals were treated with 1% Triton X-100 and ultrasound (3 strokes of 15 s each) to break the nerve terminals and centrifuged at 2,500 g for 5 min. The resulting supernatant as well as BRs, BRp, SI(s), and SIp were incubated with 1 ml of preswollen adenosine 2′,5′-diphosphate-agarose with gentle agitation (30 min at 4°C), transferred to a fritted column, and washed with five column volumes of Tris buffer (10 mM Tris chloride, pH 7.4, 0.1 or 1 mM EGTA, 0.1 or 1 µM calmodulin, 0.1 or 0.1 µM (6-5)-diphosphate-agarose with gentle strokes of 15 s each) to break the nerve terminals and centrifuged at 100,000 g for 60 min.

**NOS assay.** NOS activity was determined as the rate of conversion of L-[3H]arginine to L-[3H]citrulline (7). Putative intron sites to detect a possible DNA amplification were analyzed by nested differential PCR technique (17). After an initial denaturation at 95°C for 3 min, a "hot start" PCR was carried out in a final volume of 100 µl containing 1 mM L-arginine and 1 mM L-citrulline. Proteins from BRs, BRp, SI(s), and SIp, before and after NOS purification, were incubated in the presence of 60,000 dpm of L-[3H]arginine, 1 mM NADPH, 4 µM FMN, 4 µM FAD, 2 mM CaCl2, 1 mM calmodulin, 0.1 µM (6-5)-diphosphate-agarose with gentle strokes of 15 s each) to break the nerve terminals and centrifuged at 100,000 g for 60 min. To obtain the particulate fraction, we homogenized the resulting pellets with buffer containing 1% Triton X-100 and centrifuged them at 100,000 g for 60 min. The supernatants [particulate brain and small intestinal fractions (BRp and SIp), respectively] were then collected for further analysis.

**NOS activity.** NOS activity was determined as the rate of conversion of L-[3H]arginine to L-[3H]citrulline by 10.220.33.4 on November 6, 2017 http://ajpgi.physiology.org/ Downloaded from
66°C for 45 s, and extension at 72°C for 45 s. The last cycle was followed by an extension step at 72°C for 7 min. As negative controls, we used isolated RNA amplified without RT or oligo(dT)$_{15}$. To check the optimal sensitivity and linearity of the PCR reaction, every PCR was carried out at 25, 30, 35, and 40 cycles and a 1:100, 1:10, and 1:1 dilution of the cDNA was amplified with 35 cycles, respectively. To verify specific amplification, PCR products were digested with different restriction enzymes: Bst II (β-actin, nNOS), Bgl II (nNOS), Pst I (iNOS and eNOS), and Alw 44 (eNOS) (Boehringer Mannheim). Bst II cleaves β-actin into a 72- and a 219-bp DNA strand. Digestion of eNOS with Alw 44 results in a 106- and a 225-bp fragment, and nNOS into a 158- and a 225-bp fragment.

Quantitative analysis was carried out after agarose gel electrophoresis by positive fluorescent emission on the transilluminator, using a video densitometry system (Gel Print Workstation; MWG Biotec, Ebersberg, Germany) and image analyzing software (One-Dscan, Scanalytics, Billerica, MA) for semiquantitization (23). The relative amount of RNA was calculated as the ratio between the optical density of nNOS and β-actin corrected for the different level of molar ethidium bromide incorporation of the PCR products with different molecular weights (34). Data are presented as means ± SE and were obtained from RNA preparations from the small intestines of 10 animals (n = 10).

5'-RACE-PCR. Thermal 5'-RACE-PCR was performed as previously described (16) with minor modifications. We isolated 1 μg poly(A) RNA of rat small intestine LM-MP with the Oligotex mRNA kit (Qiagen, Hilden, Germany) and reverse transcribed it by 400 U SuperScript II RNase H$^-$RT (GIBCO-BRL) for 2 h at 45°C using 2 pmol of the antisense gene-specific primer 1 (see Table 2 for all primers) complementary to exon 3 of rat nNOS RNA (5). After removal of template mRNA by RNase H (GIBCO-BRL) treatment, excess primers were eliminated by using a PCR purification kit (Qiagen). The 3′ end of the cDNA product was poly(A)-tailed with dATP by terminal deoxynucleotidyl transferase (GIBCO-BRL). The first round of PCR amplification was performed with gene-specific primer 2 (25 pmol), sense generic primer Q (2 pmol), and sense generic primer Q$_2$ (25 pmol) under the following conditions: denaturation at 97°C for 5 min, annealing at 50°C for 2 min, and extension at 72°C for 40 min. The subsequent 29 cycles were carried out using the step-down PCR technique (19) with the following parameters: denaturation at 94°C for 30 s, annealing temperature of the initial 9 cycles at 66°C for 40 s, with a rundown of 4°C for each subsequent 10 cycles, and extension at 72°C for 3 min adding 5 s each cycle. A second round of amplification was performed for 30 cycles in a total volume of 50 μl with the antisense gene-specific primer 3 (25 pmol) and the sense generic primer Q$_2$ (25 pmol) with denaturation at 94°C for 30 s, primer annealing at 58°C for 40 s, and extension at 72°C for 3 min. To verify specific partial cDNA amplification, we analyzed PCR products by Southern blot hybridization. After the first and second rounds of PCR, amplification products were separated by 1.5% agarose gel electrophoresis, denatured, and blotted onto Hybond N$^+$ nylon membrane (Amersham) by capillary transfer. The filters were hybridized overnight at 54°C with a 5′ digoxigenin end-labeled oligomer specific for exon 3 of rat nNOS (5′-AGACCTCGATGGCAAATCG-3′) and washed twice with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS for 15 min at room temperature and once with 0.5× SSC and 0.1% SDS for 10 min at 44°C. The labeled products were detected by the DIG nucleotide acid detection kit (Boehringer Mannheim). Finally, 20-μl aliquots of the PCR products were fractionated on a 1.5% agarose gel, excised, and purified, using a gel extraction kit (Qiagen). Nucleotide sequences were deduced by cycle sequencing of both strands of the PCR products (Medigen, Martinsried, Germany).

To confirm the expression of the detected nNOS$_x$ and nNOS$_β$ splice variants, we created specific sense strand primers for nNOS$_x$ exon 1a and nNOS$_x$ exon 1b (Table 2). Because we could not detect nNOS$_Y$ mRNA by 5′-RACE-PCR, we created a heterologue sense strand primer (Table 2), based on the published nNOS$_Y$ sequence, detected in the brain of nNOS$_x$ knockout mice (8). We performed 30 rounds of PCR amplification with random hexamer (Boehringer Mannheim) primed cDNA from LM-MP and CM with an nNOS exon 5 antisense strand primer (Table 2) (denaturation at 94°C for 30 s, annealing at 58°C for 40 s, extension at 72°C for 2 min).

Table 1. Oligonucleotides of sense- and antisense-strand primers used in semiquantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>GS primer 1 (AS)</td>
<td>5′-TTCTCTGAATACGGTTGTG3′</td>
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<tr>
<td>GS primer 2 (AS)</td>
<td>5′-GGATCAACAGAATGTTGTC-3′</td>
</tr>
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<td>GS primer 3 (AS)</td>
<td>5′-CCCCACAGGTCTTGAAGAAG-3′</td>
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<tr>
<td>Q (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
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<td>Q$_1$ (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
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<tr>
<td>Q$_2$ (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
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<tr>
<td>nNOS exon 1a (S)</td>
<td>5′-GGGAGCGCAGTCTGACAGGG-3′</td>
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<tr>
<td>nNOS exon 1b (S)</td>
<td>5′-GGGAGCGCAGTCTGACAGGG-3′</td>
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<td>nNOS$_x$ exon 1S (S)</td>
<td>5′-GGTGTGGGAGGAGGAATACTT-3′</td>
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<tr>
<td>nNOS$_β$ exon 1S (S)</td>
<td>5′-GGTGTGGGAGGAGGAATACTT-3′</td>
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Table 2. Sense- and antisense-strand primers used in 5′-RACE-PCR and RT-PCR

<table>
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<th>Primer</th>
<th>Sequence</th>
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<td>GS primer 1 (AS)</td>
<td>5′-TTCTCTGAATACGGTTGTG3′</td>
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<tr>
<td>GS primer 2 (AS)</td>
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<td>GS primer 3 (AS)</td>
<td>5′-CCCCACAGGTCTTGAAGAAG-3′</td>
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<td>Q (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
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<tr>
<td>Q$_1$ (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
</tr>
<tr>
<td>Q$_2$ (S)</td>
<td>5′-CCAGTGACAGGTGACGAGGACTCAGCT-3′</td>
</tr>
<tr>
<td>nNOS exon 1a (S)</td>
<td>5′-GGGAGCGCAGTCTGACAGGG-3′</td>
</tr>
<tr>
<td>nNOS exon 1b (S)</td>
<td>5′-GGGAGCGCAGTCTGACAGGG-3′</td>
</tr>
<tr>
<td>nNOS$_x$ exon 1S (S)</td>
<td>5′-GGTGTGGGAGGAGGAATACTT-3′</td>
</tr>
<tr>
<td>nNOS$_β$ exon 1S (S)</td>
<td>5′-GGTGTGGGAGGAGGAATACTT-3′</td>
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5′-RACE, rapid amplification of 5′ cDNA ends. GS, gene-specific.
The sequence of the PCR products was determined by cycle sequencing (Medigen). It has to be considered that the exon 1b described in the mouse (8) is different from the exon 1b described in the rat (31). To avoid confusion, we used the rat terminology throughout this study, but we added the suffix α to account for the various NOS proteins originally described in the mouse.

Drugs. L-[3H]arginine (60–68 Ci/mmol) was purchased from Amersham. All other reagents were purchased from the indicated sources. L-arginine, L-citrulline, β-NADPH, N=–nitro-L-arginine methyl ester (L-NAME), THB, calmodulin, FAD, FMN, Triton X-100, adenosine 2',5'-diphosphate-agarose, leupeptin, pepstatin, PMSF, and nitro blue tetrazolium chloride were from Fluka (Neu-Ulm, Germany). EGTA, EDTA, β-mercaptoethanol, SDS, and glycine were from Merck (Darmstadt, Germany), and Dowex AG50W-X8 resin was from Bio-Rad. Acrylamide/bisacrylamide was from Roth (Karlruhe, Germany), and scintillation fluid Quickszint 212 was from Zinsser Analytic (Frankfurt, Germany).

Data analysis and statistics. Data are expressed as means ± SE; n indicates the number of independent observations in various experiments from individual preparations. The activity of NADPH diaphorase or NOS at the different treatment protocols was measured in duplicate. For multiple comparisons, ANOVA followed by post hoc test with Bonferroni correction for multiple comparisons was carried out to determine statistical differences. P < 0.05 was considered significant.

RESULTS

Differential expression of NOS mRNA in rat small intestine. RT-PCR was carried out with RNA from LM-MP, CM, and the mucosa of rat small intestine to determine the distribution of all three NOS isoforms. RT-PCR with specific primers for nNOS, iNOS, eNOS, and β-actin as an internal standard showed single bands for each cDNA at the expected size in proportion to the increase of the PCR cycles or the amount of cDNA present in the reaction. A linearity in the increase of intensity was found between 25 and 35 cycles for β-actin and between 30 and 40 cycles for iNOS and eNOS. nNOS increased linearly between 25 and 35 cycles in the LM-MP and between 30 and 40 cycles in CM and the mucosa. After 35 cycles, a linear increase of the three isoforms and β-actin was also observed in the cDNA diluted 1:100, 1:10, and 1:1 (data not shown). The mRNA quantification was reproducible, as tube-to-tube and day-to-day variation of the ratio of xNOS to β-actin and the mean values of the amplification products showed no significant difference (data not shown). The quantification of the isoforms was carried out with the 1:1 dilution and 35 cycles. The ratio of nNOS to β-actin was ~0.785 ± 0.078 in LM-MP and 0.015 ± 0.006 in CM. Even after 40 cycles of PCR, nNOS was not detectable in the intestinal mucosa. The ratio of iNOS to β-actin was 0.178 ± 0.059 for LM-MP, 0.043 ± 0.036 for CM, and 0.569 ± 0.160 for the mucosa. The ratio of eNOS vs. β-actin was 0.169 ± 0.045 for LM-MP, 0.192 ± 0.061 for CM, and 0.127 ± 0.046 for the mucosa (Fig. 1). As we were interested in the characterization of nNOS, all further studies were performed either on isolated enteric synaptosomes or on proteins or RNA extracted from LM-MP preparations.

NADPH diaphorase and NOS activity in isolated nerve terminals. Because NADPH diaphorase and NOS are present in the same protein, we used diaphorase activity [absorbance units (AU)/mg protein] and NOS activity (fmol·mg−1 protein·min−1), measured by the conversion of L-[3H]arginine to L-[3H]citrulline as parameters for the NOS content of isolated nerve terminals (21). The isolation of nerve terminals led to a 5.4-fold (n = 7) enrichment of NADPH diaphorase activity in the synaptosomal fraction (2.69 ± 0.79 AU/mg protein) compared with PNS (0.50 ± 0.19 AU/mg protein), which was paralleled by a 4.3-fold (n = 3) enrichment of NOS activity in P2 (4.6 ± 2.3 fmol·mg−1 protein·min−1; PNS, 1.1 ± 0.6 fmol·mg−1 protein·min−1). In comparison, the enrichment factor for [3H]saxitoxin binding or the content of bombesin-, somatostatin-, or VIP-like immunoreactivity was 8.2, 4.0, 3.8, and 7.1, respectively (Table 3). These experiments indicate that NOS activity can be enriched by the isolation of enteric nerve terminals.

NOS activity in isolated nerve terminals increased in the presence of the cosubstrates NADPH (1 mM) and Ca2+ (0.5 mM; P < 0.05, n = 7). Addition of calmodulin

Fig. 1. Semiquantitative analysis by RT-PCR of neuronal nitric oxide synthase (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) mRNA in the longitudinal muscle layer (LM-MP), the circular muscle (CM), and mucosa (M) of rat small intestine. Whereas nNOS is predominantly present in LM-MP, iNOS is mainly located in the mucosa. Distribution of eNOS was equal in all 3 tested layers. For semiquantification, ethidium bromide-stained gels were scanned by video densitometry and the ratio of the optical density (OD) of xNOS (nNOS, iNOS, and eNOS) and β-actin were determined. Data are presented as means ± SE; n = 10.

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Table 3. Enrichment of NADPH diaphorase and NOS activity, specific [3H]saxitoxin-binding and content of BLI, SLI, and VIP-LI in PNS and P2

<table>
<thead>
<tr>
<th></th>
<th>NADPH Diaphorase, AU/mg</th>
<th>NOS Activity, fmol·mg⁻¹·min⁻¹</th>
<th>STX Binding, fmol/mg</th>
<th>BLI Content, ng/mg</th>
<th>SLI Content, ng/mg</th>
<th>VIP-LI Content, pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNS</td>
<td>0.5±0.19</td>
<td>1.1±0.6</td>
<td>5.5±1.7</td>
<td>0.3±0.04</td>
<td>2.2±0.3</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>P2</td>
<td>2.7±0.79</td>
<td>4.6±2.3</td>
<td>44.9±8.0</td>
<td>12±0.2</td>
<td>8.4±0.7</td>
<td>6.4±1.9</td>
</tr>
<tr>
<td>Enrichment factor</td>
<td>5.4</td>
<td>4.3</td>
<td>8.2</td>
<td>4.0</td>
<td>3.8</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. STX, saxitoxin. BLI, bombesin-like immunoreactivity. SLI, somatostatin-like immunoreactivity. VIP-LI, vasoactive intestinal polypeptide-like immunoreactivity. PNS, postnuclear supernatant. P2, enriched enteric nerve terminals. AU, absorbance units.

Data for STX binding and BLI content were taken from Ref. 28. Data for SLI content are from Ref. 29, and data for VIP-LI content are from Ref. 2.

(1 µM; P < 0.05) or THB (0.1 µM; P < 0.01) alone caused a significant increase above basal levels. The combination of THB (0.1 µM) plus calmodulin (1 µM) increased NOS activity significantly compared with NADPH/Ca²⁺ (P < 0.05, n = 7; Fig. 2A). In a second series of experiments, the effect of FAD (4 µM) and FMN (4 µM) on NOS activity in the presence of NADPH, Ca²⁺, calmodulin, and THB was tested. FAD and FMN further increased NOS activity (NADPH, Ca²⁺, calmodulin, THB: 6.2 ± 1.7 fmol·mg⁻¹·protein⁻¹·min⁻¹; plus FAD and FMN: 7.1 ± 1.2 fmol·mg⁻¹·protein⁻¹·min⁻¹; n = 6, P < 0.05).

Maximal NOS activity in isolated nerve terminals obtained by addition of all cofactors could be reduced with EGTA (0.1 mM) from 5.3 ± 1.2 to 1.4 ± 0.7 fmol·mg⁻¹·protein⁻¹·min⁻¹ (P < 0.05, n = 6) or L-NAME (0.5 mM; 0.4 ± 1.1 fmol·mg⁻¹·protein⁻¹·min⁻¹, P < 0.05, n = 6; Fig. 2B) but not by N-nitro-o-arginine methyl ester (4.7 ± 0.1 fmol·mg⁻¹·protein⁻¹·min⁻¹). The NOS activity in enriched enteric nerve terminals is predominantly Ca²⁺ and calmodulin dependent, and the cofactor requirement would be in agreement with the presence of a constitutive isoform, e.g., nNOS and eNOS. There is a small residual activity present after EGTA pretreatment, which showed a marginal significant stimulation above basal levels when further investigated in additional experiments (n = 12, P < 0.05, paired t-test vs. basal). This residual NOS activity was also present at higher concentrations of EGTA (1 mM).

Isolation, purification, and Western blot analysis of NOS. For further characterization through Western blot analysis, NOS was isolated and purified from BRS, BRP, SLs, and SIp, and P2 by adenosine 2',5'-diphosphate-affinity chromatography. The enrichment of NOS activity vs. the crude fractions achieved by affinity chromatography was 133-fold for BRS (21.2 vs. 0.16 pmol·mg⁻¹·protein⁻¹·min⁻¹), 359-fold for BRP (33.5 vs. 0.09 pmol·mg⁻¹·protein⁻¹·min⁻¹), 114-fold for SLs (22 vs. 0.18 pmol·mg⁻¹·protein⁻¹·min⁻¹), and 90-fold for SIp (3.1 vs. 0.03 pmol·mg⁻¹·protein⁻¹·min⁻¹). In P2, the further enrichment was 59-fold (197.0 vs. 3.93 fmol·mg⁻¹·protein⁻¹·min⁻¹; n = 3) (Fig. 3).

Purified NOS from rat brain and rat small intestinal LM-MP and isolated nerve terminals was investigated by immunoblot analysis with antibodies for nNOS, eNOS, and iNOS. Using the nNOS antibody, which is directed against the COOH-terminal domain, we detected three bands of -155, 145, and 135 kDa in BRS and SLs as well as in P2 of rat small intestine. However, the quantitative distribution of these three bands within these preparations seemed to be different. When the relative density of the different bands at 155, 145, and 135 kDa was analyzed, a relationship of 0.72:0.07:0.21 in BRS, 0.54:0.11:0.35 in SLs, and 0.31:0.12:0.57 in P2 was detected. In SIp, only a single band at 155 kDa
could be isolated (Fig. 4). Incubation of the purified NOS with an antibody directed against eNOS or iNOS did not show a positive signal in the Western blot analysis of proteins isolated from LM-MP and isolated nerve terminals of rat small intestine. However, both antibodies resulted in a specific band with the expected molecular mass with the respective positive controls for iNOS and eNOS. This indicates that the amount of iNOS and eNOS proteins in isolated nerve terminals and the LM-MP was below the detection limit. There was no cross-reaction of the nNOS antibody with either the iNOS or the eNOS positive control.

The results of these experiments indicate that three different proteins that react specifically with an nNOS antibody directed toward the COOH-terminal end can be found in rat small intestine. These proteins, present in the LM-MP as well as in isolated nerve terminals, show distinct subcellular distribution and could suggest the possible existence of different NH\textsubscript{2}-terminal splice variants.

Detection of different 5'-end splice variants of nNOS by 5'-RACE-PCR. To differentiate the positive protein bands obtained with the nNOS antibody, we investigated possible 5'-end splice variants of nNOS. Rat small intestinal mRNA was isolated from the LM-MP, reverse transcribed, and 5'-RACE-PCR was performed. The amplified products with ~350, 1,050, and 1,500 bp were able to hybridize with an exon 3-specific internal oligonucleotide probe (Fig. 5, A and B). Sequencing of these RACE products demonstrated the existence of three different nNOS 5'-end mRNA transcripts in rat small intestine (Figs. 5D and 6). Two distinct 5'-terminal exons (exon 1a, exon 1b) located in the 5'-untranslated region of nNOS mRNA could be determined, followed by a common exon 2, which contains the translational initiation site with an ATG starter methionine, 378 bases downstream of the exon 1/exon 2 splice junction. Translation of these two splice variants should result in only one nNOS protein (nNOS\textsubscript{a}). Our exon 1a is almost identical (alignment 99.3%) to the sequence of the rat exon 1a reported by Lee et al. (31) and shows 92% alignment to the reported alternative spliced exon 1a of mouse nNOS mRNA (8). Our exon 1b is almost identical to the nucleotide sequence of exon 1b reported by Lee at al. (31) and shows 88% homology with another recently reported exon 1 subtype from the mouse brain (10). In accordance with the nomenclature for the rat by Lee et al. (31), this exon 1 splice variant is referred to as exon 1b. The third mRNA is an exon 1a/exon 3 splice variant that lacks exon 2, which encodes the PDZ domain of the nNOS\textsubscript{a} splice variant. The mRNA shows 92% identity with the 5'-end nucleotide sequence of mouse brain nNOS\textsubscript{b} mRNA reported by Brenman and co-workers (8). The translation initiation site (CTG) of this transcript is located 20 bases upstream of the exon 1a/exon 3 splice junction, and translation of nNOS\textsubscript{b} mRNA should generate an alter-

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Fig. 3. Purification and enrichment of NOS. NOS was isolated from soluble and particulate fractions of rat brain (BR\textsubscript{s} and BR\textsubscript{p}, respectively), rat small intestinal LM-MP (SI\textsubscript{s} and SI\textsubscript{p}, respectively), and enriched nerve terminals of rat small intestine (P2) by adenosine 2',5'-diphosphate-affinity chromatography. The enrichment of NOS activity vs. the crude fractions was 133-fold for BR\textsubscript{s}, 359-fold for BR\textsubscript{p}, 114-fold for SI\textsubscript{s}, 90-fold for SI\textsubscript{p}, and 50-fold for P2. Open bars, crude fraction; solid bars, purified enzyme.

Fig. 4. Western blot analysis of purified NOS from BR\textsubscript{s} (70 ng/lane), SI\textsubscript{s} (140 ng/lane), and P2 (20 µg/lane). Proteins were detected with an antibody specific for the COOH-terminal domain of nNOS. Proteins at 155, 145, and 135 kDa in the soluble fractions were present in all tissues. The band at 155 kDa could be isolated from the other bands in the particulate fraction.

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native nNOS protein (nNOSβ) that lacks the NH₂-terminus and subsequently the PDZ domain of nNOSα. The PDZ domain can interact with proteins from the postsynaptic density zone and subsequently influence the cellular localization of the enzyme. We could not detect mRNA of an alternative spliced exon 1 in rat small intestine by 5'8-RACE-PCR that corresponds to nNOS in the brain of nNOSα-knockout mice (8).

To verify the existence of alternative spliced mRNA transcripts in the LM-MP of rat small intestine, we performed a RT-PCR with rat nNOS sense-specific primers designed according to the determined sequences of exon 1a (lane 1), exon 1b (lane 2), and antisense primer corresponding to the common exon 5 confirmed the expression of the 3 identified nNOS mRNA splice variants. D: 3 different RACE products of rat nNOS mRNA were identified by sequencing, i.e., 2 different 5'-terminal exons 1 (exon 1a and exon 1b) followed by a common exon 2 and an alternative spliced exon 1a/exon 3 variant that lacks exon 2.
splice variants. Because we could not detect nNOSγ mRNA by 5'-RACE-PCR, we created a heterologue sense strand primer, based on the published exon 1 sequence of mouse nNOSγ (8); however, RT-PCR showed no specific product (data not shown). These data demonstrate that nNOSα and nNOSβ are present in rat small intestine and that nNOS originates from two different mRNA splice variants (nNOSα-a and nNOSα-b).

DISCUSSION

We demonstrated that NOS activity, as well as enzymatic diaphorase activity, was enriched in isolated nerve terminals and the enrichment of both enzyme activities showed the same relation as the enrichment of putative enteric neurotransmitters or neuronal markers. NOS activity in the rat enteric nervous system required NADPH, Ca²⁺, calmodulin, THB, and FAD plus FMN for full enzymatic activity. Kostka et al. (27) also found an increase of NOS activity in isolated nerve terminals of the canine ileum by addition of NADPH, Ca²⁺, and calmodulin; however, they could not demonstrate a further increase by addition of THB, which caused a twofold increase compared with the values with NADPH and Ca²⁺ alone in our model. The activity of NOS in isolated nerve terminals was regulated crucially by Ca²⁺, which enables calmodulin to bind and activate the enzyme since NOS activity was almost abolished by EGTA. These functional results are in accordance with the NOS isoform detected by Western blot analysis, and these cofactors were requirements for full activity of the nNOS enzyme isolated from brain (5, 6, 37). A small residual component of NOS activity was not abolished in the presence of EGTA (0.1 or 1 mM), reflecting small amounts of Ca²⁺-independent NOS activity in this fraction. This finding is in agreement with that of Kostka et al. (27), who also identified an EGTA-insensitive NOS activity in canine synaptosomes. In their detailed characterization (27), this activity was unlikely to be due to a smooth muscle cell contamination and was somewhat different from iNOS activity.

To determine the expression of NOS isoforms within different intestinal layers, we performed semiquantitative RT-PCR with RNA from the LM-MP, CM, and mucosa of rat small intestine. It could be demonstrated that NOS is predominately present in the LM-MP, whereas the inducible isoform, according to its predominant location in immune cells (20, 44), is mainly located in the mucosa and to a lesser extent in the LM-MP. The distribution of eNOS was equal in all three layers tested, as expected because of the presence of blood vessels in all of them. However, even after purification of NOS activity with adenosine 2',5'-diphosphate-affinity chromatography, Western blot analysis with specific antibodies for eNOS and iNOS could not demonstrate the existence of these latter two isoforms in isolated nerve terminals or in nerve/muscle homogenates. Despite the fact that small amounts of the enzyme could be missed by protein loss during the purification, these results indicate that eNOS and iNOS protein expression in the LM-MP and enriched nerve terminals was below the limit for detection of our Western blot analysis. This corresponds with the distribution obtained by the semiquantitative RT-PCR in the different layers as the levels of iNOS and eNOS mRNA were considerably lower in the LM-MP fraction compared with the nNOS expression. It is also in agreement with recent findings (3) in whole thickness preparations of rat stomach and rectum, in which significant amounts of iNOS were only present after upregulation with interferon-γ.

As recently demonstrated (8, 15), two different NH₂-terminal splice variants of NOS (nNOSα, nNOSβ) with different molecular masses (150 and 136 kDa) are present in the brain of wild-type mice. nNOSγ (125 kDa) was found as an additional splice form besides nNOSβ in the brain of nNOSα knockout mice (8). nNOSα exhibits full enzymatic activity and is partially membrane associated via a PDZ domain. The PDZ domain in nNOSα is a motif of ~100 amino acids that mediates an association of nNOSα to postsynaptic density proteins (e.g., PSD-95, PSD-93, syntrophins), which associate with the dystrophin complex (8, 9, 39). This association can determine the cellular localization of nNOS, and impairment of this association in skeletal muscle was shown in Duchenne muscular dystrophy (9). nNOSβ and nNOSγ, which lack the PDZ domain, are subsequently soluble and show enzymatic activities of ~80% and ~3% of the full activity observed with nNOSα, respectively (8).

Through immunoblotting with specific NOS COOH-terminal antibodies, we demonstrated that isolated and purified NOS from all three sources, BR₃, SI₃, and P2, displayed three different protein bands at ~155,
145, and 135 kDa. In SI, only the protein band at 155 kDa could be detected, corresponding to the expected molecular mass and cellular localization of nNOSα. The molecular mass of the soluble 135-kDa protein correlates with the nNOSβ variant. However, definitive proof based on Western blot analysis without specific antibodies against this splice form cannot be given. Considering the results by Lowe et al. (32), who showed a specific cleavage of nNOSα by trypsin and chymotrypsin treatment, it might also be possible that proteins with lower molecular mass arise from enzymatic cleavage of the nNOSα protein. The enzymatic cleavage at amino acid 221 could result in an NH$_2$-terminal truncated nNOS fragment with 135 kDa, devoid of the functional PDZ domain. To avoid enzymatic cleavage, several protease inhibitors also specifically directed against trypsin and chymotrypsin were used in the present study. The single protein band obtained in SI, argues against a proteolytic breakdown; however, on the basis of the immunoblot data this possibility cannot be completely ruled out.

5'-RACE-PCR with LM-MP cDNA of rat small intestine resulted in three different splice variants: exon 1a/exon 2, exon 1b/exon 2, and exon 1a/exon 3. Translation of the splice variants exon 1a/exon 2 (nNOSα-a) and exon 1b/exon 2 (nNOSα-b) would result in a 155-kDa protein corresponding to nNOSα, which is partially particulate but also occurs in the soluble fraction. This is in accordance with the possible membrane association of this splice form via the PDZ-binding domain. Translation of the exon 1a/exon 3 variant, which lacks exon 2 and subsequently the PDZ domain, would result in the soluble splice variant nNOSβ (135 kDa). nNOSα is the predominant form in the brain of wild-type mice, whereas nNOSβ and nNOSγ account for NOS activity in nNOSα knockout mice (8, 15). In the rat, both nNOSα and nNOSβ are present in the small intestine. However, in contrast to rat brain, the relative expression of nNOSβ seems to be increased in LM-MP and predominant in isolated nerve terminals of the intestine. The functional roles of nNOSα and nNOSβ remain speculative. In myenteric plexus of embryonic rats, nNOSα and PSD-95, a protein with specific PDZ motifs that interacts with nNOSα, are colocalized (8). PDZ motif-containing proteins (e.g., PSD-95) were suggested to be involved in the subcellular localization of proteins (e.g., N-methyl-D-aspartate receptors, K+-channel subtypes) at sites of membrane specialization such as synaptic sites (25, 26). Thus the membrane-associated form (nNOSα) could be involved in the regulation of receptor or ion channel function. The role of nNOSβ that is not membrane associated but is expressed relatively high in rat small intestine, especially in enriched nerve terminals, has yet to be determined. If a comparable distribution of splice variants is present in mouse intestine, it would explain why nNOSα-knockout mice, lacking only nNOSα, show no major functional abnormalities in the small intestine (24). Whether the nNOS splice variants are confined to neurons or can also occur in intestinal smooth muscle (12) or interstitial cells of Cajal (33, 49) cannot be determined from our study.

We could further demonstrate in rat small intestine that nNOSα originates from two different splice variants, which are almost identical to splice variants of the 5'-untranslated region recently described in the rat (nNOSα-a, nNOSα-b) (31) and show sequence homology to exon 1 splice variants from mouse brain (8, 10). Because translation of both exon 1 variants in rat small intestine should result in an identical nNOSα protein, the question about the physiological significance of these exon 1 splice variants arises. Lee et al. (31) demonstrated a distinct tissue and developmental-specific expression of three 5'-untranslated region exon 1 splice variants in the rat. However, in contrast to our study, Lee et al. (31) could only demonstrate the exon 1b/exon 2 splice variant (nNOSα-b) in embryonic tissue. Xie et al. (47) demonstrated that two distinct first exons of nNOS of human cerebellum are under the transcriptional control of two closely linked, but separate promoters. Transcriptional control by separate promoters and regulation of these promoters may result in expression of nNOSα in response to different cellular changes or developmental stages (10, 31, 47). The differences in first exon structure of nNOSα may also be implicated in mRNA localization, stability, posttranscriptional control, or translation efficiency (10).

The immunoblot reactive band at 145 kDa could not be further characterized by 5'-RACE-PCR, indicating that this band is not due to an NH$_2$-terminal splice variant. In particular, the molecular size of the band does not correspond to nNOSγ (125 kDa), which was also not identified by 5'-RACE-PCR and RT-PCR. Thus our results comply with the results in the mouse brain (8) in which nNOSγ was also absent in wild-type mice and was only expressed when nNOSα was knocked out.

In summary, nNOS activity is present in enteric neurons and can be purified along with other neuronal markers in enteric nerve terminals. The nNOS in enteric synaptosomes exhibits immunologic and biochemical properties identical to those of the nNOS from rat brain. Three different splice variants of nNOS are present in the small intestine, resulting in two distinct proteins that differ in their NH$_2$-terminal ending and show a distinct subcellular distribution. From our data there is no evidence for the expression of the nNOSγ form in rat small intestine.

Expression of nNOS splice variants could be changed under developmental but also physiological or pathophysiologic conditions. Because of the differences in transcriptional control, enzymatic activity, and subcellular localization, these splice variants could also be involved in functional changes. Thus, in addition to transcriptional regulation of gene expression as previously shown in diabetic rats (45), alternative splicing of nNOS has to be considered in functional disorders also.

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REFERENCES